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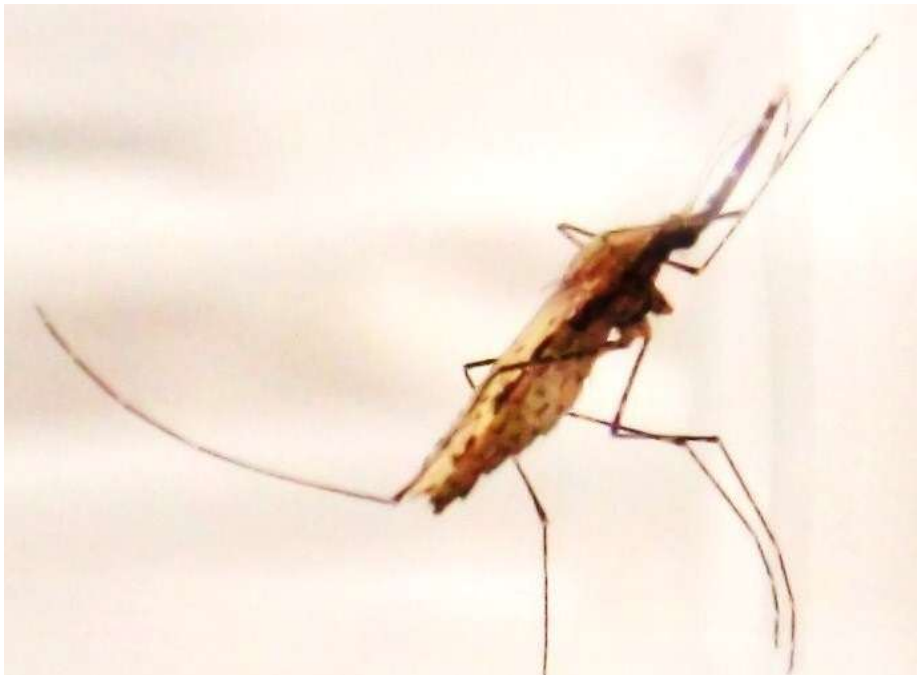
Research project 2: 36 ECTS
Masters of Biological sciences-track Ecology and Evolution
January-June 2013



Repellent, irritant and toxic effects of plant EO compounds on the malaria vector *Anopheles gambiae ss Giles*

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Female *Anopheles gambiae* photographed by C. Montazeau, IRD, 2013

Abstract

Pyrethroids resistance has become widespread among malaria vector *Anopheles gambiae* populations in Africa. Alternative insecticides must be considered to prevent diseases transmission by *An. gambiae* bites. The long lasted treated bed nets are particularly efficient to prevent blood meals on humans and thus to reduce the risks of transmissions. Previous studies have identified plant essential oils (EO) as a potential alternative to pyrethroids. Four of them showed significant repellent, irritant and/or toxic effects against *An. gambiae*.

The objective of this study was to evaluate the repellent, irritant and toxic actions of the major compounds of these four EO from citronella (*Cymbopogon winterianus*), cinnamon (*Cinnamomum zeylanicum*), cumin (*Cuminum cyminum*) and thyme (*Thymus vulgaris*), against females *An. gambiae.* We tested the spatial repellency, the contact irritancy and the toxicity effects of each product with laboratory tests over 180 females, for each action tested. The complete EO, the mixture of the major compounds (>3%) and the two currently used repellent (DEET) and insecticide (Permethrin) were also tested. The most promising compounds were similarly tested with two strains of *An. gambiae*: resistant to pyrethroids (KdrKis) and resistant to carbamates (AcerKis). At last assays in tunnels to simulate field conditions were made to determine their efficacy when a blood meal was offered.

Carvacrol, geraniol, cuminaldehyde and cinnamaldehyde showed the best repellent and irritant effects among all products tested. Cuminaldehyde and cinnamaldehyde revealed to be more efficient against the resistant strains than the susceptible strain of *An. gambiae*, and with carvacrol, they were particularly efficient to inhibit blood meals in simulated field conditions. The study also showed interested characteristics of plant EO which led to new research directions to use plant EO for biological control. At last this study was the first to test the efficiency of DEET on resistant *An. gambiae* strains.

Keywords: *Anopheles gambiae*, *kdr*, acetylcholinesterase, DEET, *Cymbopogon winterianus*, *Cinnamomum zeylanicum*, *Cuminum cyminum*, *Thymus vulgaris*, EO, repellency, irritancy, toxicity, vector control.

Introduction

The complex *Anopheles gambiae sl.* (Diptera: Culicidae) consists of at least seven morphologically indistinguishable species. Among them, A*nopheles gambiae ss Giles* (1902) is the most efficient vector of malaria parasite *Plasmodium falciparum* , mostly because of its high anthropophily [1, 2]. Five chromosomal forms are distinct in *An. gambiae ss* populations, designated by a non- Linnean nomenclature: Bamako, Mopti, Savanna, Forest and Bissau [3, 4] . The two genetic variants, referred to as the molecular forms M (Mopti) and S (Savanna) are found in *An. gambiae ss* [5-7]. But these two diverging forms are still considered as one species [3, 8, 9]. Both forms are anthropophilic and effective vector for Malaria in Africa. Unfortunately, they do not respond similarly to vector control methods and both S [10-12] and M forms [13] have resistant populations to the most common used insecticides, some particularly resistant to one type of insecticides while others can show multiple insecticide resistance [14, 15].

Among the current methods to prevent malaria transmission, one is the protection against mosquito bites. To effectively prevent mosquitoes bites, physical barrier associated with chemicals, defined as long lasting insecticide treated bed nets (ITB), are supposed to reduce the vector-host contact. Pyrethroids are the only insecticides that are used on bed nets, because they are relatively safe for humans and their rapid excito-repellent, knockdown and killing effects [16]. Pyrethroids are synthetic molecules derived from pyrethrum, extracted from *Chrysanthemum cinerariaefolium.* They modify the inactivation kinetic of the Na channel: they keep the Na channels from closing regularly, creating a succession of random action potential. This provokes uncoordinated movements, knock down effects and death of insects [17]. Other wide used insecticides for public health are the organophosphates (OP), insecticides derived from phosphoric acid, and carbamates (CX), synthetic insecticides derived from eserine. These two families inactivate the acetylcholinesterase enzyme (AChE; EC3.1.1.7), responsible for neurotransmitter degradation at the cholinergic nerve synapse [18]. The inhibition of AChE provokes the accumulation of acetylcholine in the synaptic junction. When the concentration in acetylcholine is too high, the receptors remain opened inducing paralysis and death. An insect repellent to mention is the N,N-diéthyl-3-méthyltoluamide (DEET) because it is one of the most used as it is efficient against all mosquito species [19-21]. DEET is known to block responses of olfactory neuron receptors and therefore masking attractive odours in *An. gambiae* and *Drosophila melanogaster* [22, 23]. It was also recently found to be AChE inhibitor in mosquitoes as well as humans [24].

In *An. gambiae sl* from West Africa, resistance to pyrethroids and other insecticides used for public health has been reported [10-12, 25-28]. A common form of resistance to pyrethroids and dichloro-diphenyl-trichloroethane (DDT) in *An. gambiae s.l.* called *knock-down resistance (kdr*) resulted from a mutation (L1014F) of the voltage-gated sodium channel (VdpNaC), the target site of these insecticides [13, 25, 29]. This mutation is characterized by a reduced affinity of the pyrethroids for the VdpNaC [30]. The *kdr* gene has been predominantly reported in the molecular S form of *An. gambiae ss*. [10-12]. Similar mutation but involving other amino acid substitution at the same position were found spreading among other Anopheline populations across Africa [31-34]. A L1014S *kdr* mutation primarily detected in east Africa was also identified in *An. gambiae ss* populations in Burkina Faso [34, 35]. Alternative insecticides were thought to be used in replacement for pyrethroids that are becoming inefficient against these resistant populations, and mixture between other insecticides and pyrethroids were tested [36]. CX and OP were thought to be good alternatives, to be used alone or in combination with pyrethroids, unfortunately, resistance against CX and OP is also spreading across Africa [37]. A mutation of the AChE-1 (Gly119Ser) on the *AChE ace-1* gene, responsible for the reduced sensitivity of AChE1 to CX and OP, was detected in *An. gambiae* from Cote d'Ivoire [37, 38] and other *An*. species [39]. Even multiple insecticide resistance was found in *An. gambiae* populations [14, 26]. Genetic resistance to DEET also started to appear in some populations of *Drosophila melanogaster* [40] and *Aedes aegypti* [41]. The fact that DEET can act on mammalian AChE1 and was suspected of neurotoxicity in humans, when combined with other insecticides [24, 42], strengthens the need to search for alternatives to chemical insecticides and repellents.

The pest management engaged against disease vectors and agricultural pests is facing challenge in developing suitable compounds to repel or kill insects, while ensuring economic and ecological sustainability [43]. The growing demand for natural products has increased in the past decade as it is considered as biological alternative strategies for sustainable pest control. They must be biodegradable and potentially suitable for use in integrated management programs.

Plant essential oils (EO) are mixtures of 10 to 60 hydrophobic molecules, in various proportions, volatiles and characterized by a strong odor. They are mainly terpenes, terpenoids and aromatic compounds [44], formed by aromatic plants, as secondary metabolites [45] and are involved either in pollinator attraction, or protection against herbivores, bacteria or fungus. Two or 3 of the major compounds of these EO, present in high concentrations (20-70%) are usually responsible for the biological properties of the complete EO. More than 3,000 EO have been identified and 300 of them are already commercialized. Despite their wide use and being familiar to their fragrance, it is important to develop a better understanding of the biological actions of EO to be able to consider them as effective alternatives to current insecticides used in pest and vector management.

Some EO have already been investigated for their insecticide properties (ie *lavanda ssp mentha ssp Thymus vulgaris*)[46]. For instance, Thyme EO contains thymol that is was active on the GABA gated chloride channel [47].The toxicology of a few EO and their compounds were also tested on standard organisms [45].It was found that thanks to their lipophilic properties, they cross cell barriers and disturb the normal cell functions, that could lead to toxic effects. For instance, tea tree oil would induce cytoplasm coagulation in *Escherichia coli*, leading to cell death [48]. Some EO has showed also repellent actions on *Aedes spp* [49, 50]. Because of their various mode of action (ie: behaviour modification, toxicity), EO represent interesting alternatives to currently used insecticides. EO as a combination of several active ingredients could affect several targets at the same time and therefore, neither resistance nor adaptation to these product has been yet described [45].

The discovery, development and use of novel vector control tools are required to achieve the goal of malaria eradication. Other approaches than lethal ones to interrupt human-vector contact have to be considered. However, the adoption of new methods, which aim to reduce risks of insecticide resistance, would require a new set of laboratory and field assays which have to be conducted under standardized procedures and analyses. They must be approved and adopted by leading global public health authorities. A new vector management program would consist in repelling mosquitoes instead of killing them [51]. The current vision is that toxic compounds have the greatest impact for disease prevention because it decreases the survival of the overall vector population; a concept based on the Ross-Macdonald model [52]. Also, behavior modifications such as deterrent effects are dramatically reducing malaria transmission [53].Indeed, malaria transmission can be reduced if mosquitoes are diverted to alternative hosts to human for their blood meal and if the reproduction and survival of mosquitoes decreases because of the difficult access to blood meals [54].

The actions of the insecticides which aim to reduce the host-vector contact are spatial repellency and contact irritancy [55]. The three actions: spatial repellency, contact irritancy and toxicity have to be tested to identify the range of actions of a chemical. Chemicals can have either one of the actions, either a combination of two or three of these actions [55, 56]. Spatial repellent response corresponds to the oriented movement of the mosquito away from a chemical source without direct contact with the chemical [57]. The contact irritant response is defined as the oriented movement of the mosquito away from a chemical source after direct contact with the chemical [55]. The toxicity effect of a chemical is defined by its poisonous or harmful effect on the mosquito after a direct contact with the product and within 24hours after exposure. In this study, toxicity refers to the killing effect of the product.

By deterrent actions, the compounds would modify vector behavior that result in movement away from human hosts. The immediate advantages are that vector contacts with insecticides are diminished and therefore, insecticide resistance emergence is delayed or reduced [51]. Spatial repellency would minimize the intensity of selection pressure from contact mediated toxicity mechanisms and would also potentially reduce the toxic effects of chemicals to non-target organisms [51]. Spatial repellents may show permanent or semi-permanent disruption for host seeking and blood feeding behaviors on human hosts [49]. Vector population decrease and mortality would not be directly induced and without chemically induced selection pressure, hereby ensuring the sustainability of novel chemicals used. The

discovery and development of new chemicals which target the vector behavior outside and surrounding hosts habitations would be a promising objective for malaria prevention.

Assembling novel ideas of vector control by behavior modification and plant originating compounds are the goals of the research program NatProtect, which aim to identify natural products to be used against agricultural pests and disease vectors. This project is simultaneously done by two research teams at Cirad and IRD (Montpellier, France). All experiments presented here were performed at the World Health Organisation (WHO) collaborating center: Laboratoire des Insectes Nuisibles (LIN), part of the IRD research unit MiVEGEC (IRD UMR224, Montpellier, France).

The first step of this research program was to identify among 20 EO, which ones would show the most promising repellent, irritant and toxic actions against the females of the susceptible reference strain *An.gambiae ss*, called Kisumu. Selected plants originated from Africa and Europe and were selected because of their ancient use to repel biting insects and agricultural pests (Deletre et al submitted.). The behavior of females *An. gambiae ss* towards the various EO was assessed with a High Throughput Screening System (HITSS)[58] and WHO standardized procedures [82]. Originally designed to evaluate the behavior of *Ae. aegypti L.*in presence of odors [55, 58, 59], HITSS consisted in attracting females through a treated chamber, using light. As *An. gambiae* females did not respond similarly to light attractant as did *A. aegypti L*, the system had to be adapted to *An. gambiae* (Deletre et al. submitted).

Four EO revealed excellent repellent effects on *An. gambiae: Cymbopogon winterianus* (citronnella), *Cuminum cyminum* (cumin), *Cinnamomum zeylanicum* (cinammon) and *Thymus vulgaris L.* (thyme). Their composition was characterized by gas chromatogram (GC) and gas chromatogram and mass spectrometry (GC-MS) (see Table 2, supplementary material). As EO are a mixture of various chemicals, it was unclear which compound of these EO was efficient and which role (ie. toxic) was associated to it.

The study presented here follows the previously presented project and aimed at identifying EO compounds as potential alternative products to pyrethroids, to be used on bed nets. The aims of this study were to 1) identify which compounds were responsible for the repellent, irritant and /or toxic effects of citronella, cumin, cinnamon and thyme EO previously identified, against females *An. gambiae* from the reference susceptible Kisumu strain; 2) test whether the promising compounds identified with Kisumu, were also effective against females *An. gambiae* KdrKis resistant strain and against the AcerKis resistant strain; 3) test whether these products are efficient in simulated field conditions, when applied on a net with the presence of a bait (*Cavia porcellus*, Guinea pig), against Kisumu.

Material and method

Insects:

Three reference strains of *An. gambiae ss* (S form) were used in this project:

- An insecticide susceptible reference strain ‘’Kisumu’’ (Kis), collected in 1953 in Kenya, Africa, was used in the first and the third experiments. The insecticide susceptibility of this strain was confirmed with WHO diagnostic doses (i.e. 4% DDT, 0.75% permethrin) and is controlled every 4 months.
- A strain with *kdr* mutation (L1014F) and with the same genetic background than Kisumu (KdrKis), constructed in 2011 in Benin, Africa
- A strain with *ace.1* mutation (G119S) but with the same genetic background than Kisumu (AcerKis), constructed in 2006 in Benin, Africa were used in the second experiment (see Table 2, below).

Table 1 : Characteristics of the 3 *Anopheles gambiae ss* strains used in this study.

***Anopheles gambiae ss* (savanna strain):**	**Name**	**Origin (Africa)**	**Insecticide resistance**	**Mutation**	**References**
Kisumu	Kis	Kenya, 1953	Insecticide susceptible reference strain (except for dieldrin)	None	[82]
Acetylcholinesterase (AchE) insensitive Kisumu	AcerKis	Benin, 2006	Carbamates and organophosphates resistant strain	*AChE ace.1 gene*: G119S	[18, 37]
Knock down resistant (kdr) Kisumu	KdrKis	Benin, 2011	Pyrethroids and DDT resistant strain	*Kdr gene* : L1014F	[10-13]

The three *An. gambiae* populations were bred at the LIN-MiVEGEC (IRD UMR224, Montpellier, France). The susceptible and resistant populations were reared separated insectaries at 27°C +/-2°C and 80% +/- 10% RH with a photoperiod cycle 12hLight:12hDark. Anopheline larvae were reared in 3L trays with fresh water and fed with fish food (TetraMin). Emerged mosquitoes were mechanically aspirated and transferred into cubic cages (dimensions 25 cm*25cm*25cm) with Texinov nets and were fed with 10% honey-water solution until testing.

Two to 5 days old and non-blood-fed females *An. gambiae* ss. were used in this study for the experiments 1 and 2. 6-7 days old non-blood-fed females were used in the third experiment. The number of females used in each spatial repellency assay (20 females), each toxicity assay (20 females) and each contact irritancy assay (10 females) was based on previous experiments that determined the sample size required for statistical power optimizing the smallest number of replicates with the least difficulty in visual observation and with the least manipulating time (Deletre et al. unpubl).

Chemicals:

Tests were done with the four plant EO: citronella (Nactis, France), cumin (Ipra, France), cinnamon (Nactis, France), thyme (Huiles & sens, France) DEET (Sigma Aldrich, France; CAS: 134-62-3) and permethrin (Sigma Aldrich, France; CAS 52645-53-1) were used as positive controls.

The individual compounds for which proportion was >3% in the four EO were also used in laboratory bioassays:

- From citronella EO: citronellal (CAS 106-23-0), geraniol (CAS 106-24-1), citronellol (CAS 106-22-9), limonene (CAS 5989-54-8) and geranyl acetate (CAS 105-87-3);
- From cumin EO: cuminaldehyde (CAS 122-03-2), β-pinene (CAS 18172-67-3), γ-terpinene (CAS 99-85-4) and *p*-cymene (CAS 99-87-6);
- From cinnamon EO: trans-cinnamaldehyde (CAS 14371-10-9), 2-metoxy-cinnamaldehyde (CAS 1504-74-1) and cinnamylacetate (CAS 103-54-8);
- From thyme EO: thymol (CAS 89-83-8), carvacrol (CAS 499-75-2), α-terpinene (CAS 99-86-5), linalool (CAS 78-70-6) and β-caryophyllene (CAS 87-44-5).

DEET, permethrin, the four plant EO and four mixtures of the major EO compounds: citronella mix (citronellal, geraniol, citronellol, limonene and geranyl acetate), cumin mix (cuminaldehyde, β-pinene, γ-terpinene and *p*-cymene), cinnamon mix (cinnamaldehyde, 2-metoxy-cinnamaldehyde and cinnamylacetate) and thyme mix (thymol, carvacrol, α-terpinene, linalool and β-caryophyllene) were diluted at 0.1% and 1% (v/v or w/w) in a solvent constituted with ethanol (2/3) and silicone oil Dow Corning 556 (1/3). Acetone was used with permethrin instead of ethanol, because they were not miscible.

All major compounds were tested at the concentration found in the EO at the lowest efficient concentration (concentration C2) and 1/10 of this concentration (concentration C1) (Deletre et al. submitted). For instance, citronellal represents 34.7% of the citronella EO. The citronella oil was efficient at 1% so the citronellal was tested at C2= 0.35% (0.03 mg/cm²) and 10 times less: C1= 0.035% (0.003 mg/cm²). Each mixture was created with the compounds in their respective proportions in the EO. By doing this, the quantity of a compound was the same when the EO, the mix and the compound alone were tested. Each assay was preceded by a negative control in which only the solvent ethanol-silicone oil was tested (refer to Table 2, supplementary material).

Impregnated papers with the products to be tested were prepared with a standardized protocol following the World Health Organisation for Pesticides Evaluation Scheme (WHOPES) procedure [82], described below. 3.3ml of solution to be tested are homogenously deposited (with a 5ml pipette) on 0.03m² (10cm x 30cm) of chromatograph paper (Whatman, CHR1) for the spatial repellency assay. 2ml of solution are homogeneously deposited on 0.018m² (12cm x 15cm) chromatograph paper for the contact irritancy and toxicity assays. The paper must be hold with gloves to avoid pollution by skin particles or odors. The paper sizes were chosen to completely cover the inner surface of the cylinders (material described below). Each paper was used for three replicates in the spatial repellency test and the contact irritancy test. The paper was used only once in the toxicity test. The tests were made once the papers were dry (after 30min), and all replicates were done the same day. The papers were prepared at the beginning of each testing day and kept in aluminum sheets until use. Tests were made between 10am and 6pm, at 25°C +/- 1°C, 50%+/-10% RH. Each product was tested separately (one chemical a day) to prevent from any contamination. For each product tested, the control was first tested then the lower concentration, to finish with the highest. The material was washed overnight between products in a detergent solution TFD-4 (Franklab, France), concentrated at 20% for the parts in contact with the treated paper and at 10% for the other parts. The material was rinsed and dried before new tests.

Experiments 1 and 2: bioassays

The first experiment consisted in testing the repellent, irritant and toxic actions of the four plant EO, the four mixtures of the major compounds and the 17 compounds alone, on the Kisumu strain. Concentrations C1 and C2 were tested every time. In the second experiment, only the four most promising compounds were tested on the two resistant strains KdrKis and AcerKis at both concentrations C1 and C2. The two positive controls DEET and permethrin were tested in both experiments, also at both concentrations. The three following tests: spatial repellency, contact irritancy and toxicity were done under fume hood at 25°C, 47%RH. Spatial repellency tests were made with HITSS (refer to Introduction) and contact irritancy and toxicity tests were performed following standard WHO tests kits, following the WHOPES procedures [82].

Spatial repellency test: the spatial repellency assays were done using the following material from the HITSS: Two cylinders (called chambers) made of Plexiglas which insides are connected by a butterfly valve: a small door that can be opened and closed from the outside (see Photograph 1a, supplementary material). The treated chamber was covered on the inside by a chromatogram paper, impregnated with the test solution (or only the solvent for the control). The paper was covered with metallic net, to prevent direct contact between the chemical and the females. The untreated chamber was also covered with a paper, but without any solutions to have similar luminosity in both chambers. 20 females were inserted, using mechanical aspiration, into the treated chamber. After acclimatization for about 30s, the valve was opened and the mosquitoes were free to fly between both chambers. The system was kept horizontally without luminosity variation. After 10 min, the valve was closed and the females were anaesthetized with CO2 and counted in each chamber before to be removed from the system. The assay was performed three times, so 60 females were tested for each control and each concentration. The chambers were disassembled for aeration between each replicate. The assay was validated when there was less than 20% escaped mosquitoes in the control. The number of females that was found in the untreated chamber was characterized as the number of females that escaped from the product. Higher was this number, higher was the repellent effect of the product tested.

Contact irritancy test: the irritancy tests were done with smaller tubes than spatial repellency tests, corresponding to the WHO diagnostic test kit and with 10 females instead of 20. However, to keep the same number of 60 females per control and each concentration), 6 replicates were done instead of 3. The material consisted in 2 tubes, which insides were connected via a guillotine valve. One of the tubes contained the treated paper with solution of the product to test or only the solvent (control) and the other contained a paper without any product, to have similar luminosity in both tubes (see Figure 1, supplementary material). 10 females were mechanically entered in the treated tube by the door, before to connect the second tube. After 30s, the door was opened and the females had free access to both treated and untreated tubes for 10 minutes. The system was kept horizontally and without luminosity variation. The door was finally closed and females in both tubes were anaesthetized with CO2 and counted. The tubes were disassembled for aeration between each replicate. The assay was validated when there were less than 20% of escaped mosquitoes in the control. The number of females that was found in the untreated chamber was characterized as the number of females that escaped from the product. Higher was this number, higher was the irritant effect of the product tested.

Toxicity test: the toxicity test was performed using the WHO diagnostic test kit (see Figure 1, supplementary material). 20 females were mechanically inserted into the treated tube where they were in forced contact with the paper impregnated with the test solution or only the solvent (control), for one hour. The system was set up vertically during the test. After the assay, females were transferred into a untreated tube with a cotton soaked with 10% sucrose solution and were kept at 25°C, 85%RH to monitor 24-h mortality rate. The toxic effect of the product was characterized by the proportion of dead mosquitoes 24h after exposure. The assay was repeated three times to have at least 60 females tested. The assay was validated when there were less than 10% of dead mosquitoes in the control.

Experiment 3: Tunnel assay

Net impregnation: For the tunnel test, polyester nettings (0.25m x 0.25m) were pierced with 9 holes of 1 cm diameter were impregnated with 3.5ml of solutions to be tested (5.6 µl/cm²) and let 30 min to dry under fume-hood. The quantity of ethanol corresponds to the specific absorption capacity of the net according to WHOPES procedure [82]. First tests were run with permethrin, geraniol, carvacrol, cuminaldehyde and cinnamaldehyde at concentrations C2.

Preliminary assays of lasting effect of compounds: in a first step we determined how long the product stays efficient on a net, before the tests in the tunnel experiment, which lasts for 8 hours. The same device used for contact-irritant effect was used in these assays. The paper was replaced with by polyester netting (0.17m x 0.20 m), impregnated with 1.9 ml of product to be tested: carvacrol, geraniol, cuminaldehyde and cinnamaldehyde at concentrations C2 and the small pieces of net closing the tubes were also impregnated with product. The net were allowed to dry for 30 min before the first test. The net was inserted into a WHO test tube to perform a contact irritation test (see above).

Tunnel test: This experiment was meant to determine the efficacy of the product on a net in presence of a bait (guinea pig). This device allowed us to test our product in simulated field conditions, following WHOPES procedure [82]. The device consisted in a 0.25m x 0.25m x 0.75m glass tunnel divided in two chambers of length 0.25m and 0.5m; separated by a 0.20m x 0.20m mosquito net in which 9 holes of 0.1m diameter were pierced: the small chamber contained a guinea pig (meal chamber) and female mosquitoes were inserted in the larger chamber. Two cages (0.25m x 0.25m x 0.25m) were positioned at both sides of the tunnel to offer a resting place to the females away from the net (see Figure 2, supplementary material). 2 replicates were done for each product tested, on two separated days. One replicate included four tunnels: untreated control, permethrin and two products, tested the same day at the same time. About 100 unfed and 6-7 days old Kisumu females were inserted in the lager chamber. After 8 hours, the net is covered with a sheet of paper to stop the experiment. Mosquitoes alive, dead, fed and unfed were collected and counted for each chamber. The assay was validated when there were less than 20% dead and more than 60% fed females in the untreated control. The tests were done in the dark at 27°C and 80%RH, with a wind flow of approximately 0.1m/s in the tunnel, flowing from the guinea chamber to the mosquito chamber. A negative control (untreated), a positive control (permethrin) and two compounds (geraniol and cinnamaldehyde) were tested at the same time. The experiment was conducted two times for a total of at least 200 females tested per product. The two other compounds (carvacrol and cuminaldehyde) were tested at the same time and two replicates were also done.

Statistics:

All statistical analyzes were done using the free software R2.12.2 (R Development Core Team, 2012). The proportions of dead mosquitoes (toxicity assays) and escaped mosquitoes (repellency and irritancy assays) were analyzed with the same method. Data were all corrected with Abbot's formula [60] to distinguish the effects of the treatments from those caused by natural factors.

The proportions of dead or escaped mosquitoes in the different treatments from the first and second experiments and the proportions of females that crossed, took a blood meal, or died in the third experiment were compared with their controls using Fisher's exact tests. The obtained *P*-values were corrected with the Holm's sequential Bonferroni method[61] to take into account the number of tests. Also Abbot's corrected proportions were used to compare the effects of the compounds with the effects of the EO they came from, in the first experiment and to compare the proportions of repelled, irritated and killed resistant females to the proportions obtained with Kisumu females, in the second experiment. These proportions were also compared with Fisher exact tests and corrected with Holm's sequential Bonferroni method. A 95% confidence interval level was used for all analyses and confidence intervals were calculated with the central limit theorem (also called Wald's interval):

$$P \pm z_{1-(\alpha/2)} \sqrt{[(P_s / n)*(1- (P_s / n)) /n]}$$

The confidence level is $\alpha = 0.05$; P is the proportion of individuals in the trial, P_s is the number of individuals that stayed or were alive in the trial and *n* is the number of individuals tested in the trial.

For each product and each concentration, in the first experiment, the Abbot's corrected proportions were used to perform a principal compound analysis (PCA). Then, a hierarchical ascendant classification (HAC) based on Ward's algorithm was computed in order to group the plant extracts based on the similarity of their effects using PCA-axes coordinates. This process yielded a binary segmentation tree, which reflects the hierarchy of similarities between responses to compounds. The optimal number of classes was determined by the decrease of the interclass variance (branch height) to choose the optimal number of classes. We chose the four products the most promising to be used in the second and third experiments based on the classifications given by the PCA for each effect, repellency, irritancy and toxicity.

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Results

Experiment1:

The first aim of this study was to identify which EO compounds were responsible for repellent, irritant and /or toxic effects of plant EO of citronella, thyme, cumin and cinnamon, against females *An. gambiae* from the susceptible Kisumu strain (Refer to Figure 2, 3 and Table 2, supplementary material).

Repellency assay:

The four EO tested were significantly repellent when tested at C2= 0.1mg/cm^2 ($P<0.001$). The least efficient of them was cumin oil, which repelled only about 38% more females than control. Thyme and citronella oil repelled about 50% more females than control. The most efficient was cinnamon EO which repelled about 84% more females than control. The mixture of major compounds of each EO but citronella EO showed similar repellency effects than EO when tested at the same concentration. According to the similarities in behavioural responses of females *An. gambiae*, the hierarchiral clustering arranged the compounds into 4 classes. 3 classes assembled the products which did not show any significantly repellent activity on females *An. gambiae*, at the exception of cuminaldehyde, which with 14.3% to 36.5% escaped females, was significantly repellent at C2. One class assembled the product which were the most repellent at the highest concentration tested C2 ($P<0.01$): carvacrol, citronellal, cinnamaldehyde and geraniol. None of the compounds were repellent at C1. The repellent effects ranged from about 25% (carvacrol) to 63.1% (geraniol). These compounds but cinnamaldehyde had similar repellency activities on females than their originating EO.

Irritancy assay:

Permethrin significantly irritated 31.1% to 55% females *An. gambiae* compared to control at C1=0.001ul/cm^2 ($P=0.004$) but was not irritant at C2. On the contrary, DEET significantly irritated females at C2, with 42.6% to 65.7% escaped females ($P<0.0001$) and was not significantly irritant at C1. The four EO tested were significantly irritant to females compared to controls, at the highest concentration ($P<0.0001$). Their efficiency ranged from 49% (thyme EO) to 95.2% (cinnamon EO) escaped females. The mixtures of the major EO compounds were significantly irritant at both concentrations, ranging from 23% (thyme mix) to 67% (cinnamon mix) escaped females at C1 ($P<0.01$) and ranging from 26.6% (thyme mix) to 90.2% (citronella mix) escaped females at C2 ($P<0.05$). All mixtures but cumin mix (significantly less irritant than cumin EO) had similar irritancy effects than the EO they originated from. According to the similarities in behavioural responses of the females, the hierarchiral clustering arranged the compounds into 3 classes. One of these classes assembled all compounds which showed significant behavioural response of the females ($P<0.05$): the most efficient was carvacrol, up to 91.2% escaped females, then citronellol, cuminaldehyde, cinnamaldehyde, geraniol, citronellal, thymol and cinnamyl acetate, the least efficient with 32.6% escaped females. The behaviour responses of females were similar when these compounds and their originating EO were tested.

Toxicity assay

Permethrin was significantly toxic to Kisumu females of *An. gambiae* at both concentrations with mortality rates of 89.7 % to 96.8% ($P<0.0001$). They were no differences between the two concentrations. DEET was significantly toxic to females when tested at C2 ($P<0.0001$) with mortality rates of 98.2%. The four EO were significantly toxic at the highest concentration tested ($P<0.05$) with mortality rates ranging from about 34.4% for cumin EO, to about 75.9% for thyme EO. Only the mixture of the major compounds from thyme EO and cinnamon EO were significanty toxic to females ($P<0.0001$). Thyme mix, had similar mortality rates than the thyme EO, and cinnamon mix was significantly more toxic than cinnamon EO, with mortality rates up to 87.3%. According to the similarities in behavioural responses of females, the hierarchiral clustering arranged the compounds into 3 classes. One of the classes included only permethrin, which was efficient at both concentrations. The second class regrouped DEET, cinnamaldehyde and citronellal, which were toxic at C2. Cinnamaldehyde was the only compounds which was significantly toxic to females with mortality rates ranging between 32.9% and 58.8% ($P=0.001$). Citronellal was not significantly toxic despite mortality rates up to 32.2%. The last class assembled the other compounds, which were not toxic to females.

Experiment 2:

According to the behavioral responses of female Kisumu obtained toward repellency, irritancy and toxic effects in the first experiment, we selected the most efficient compounds: geraniol, carvacrol, cuminaldehyde and cinnamaldehyde. The second part of this study was to test these most promising compounds on two resistant strains of *An. gambiae* KdrKis (resistant to pyrethroids) and AcerKis (resistant to CX and OP), to determine if there was cross resistance with some of the compounds (refer to Table 3, supplementary material).

Repellency assay:

Permethrin did not repel females *An. gambiae* when tested at both concentrations. DEET induced a significantly behavioural response of females from both resistant strains, with a range of 17.4% to 40.9% repelled KdrKis females and from77.6% to 93.8% escaped AcerKis females. These last percentages obtained for the AcerKis females were significantly higher than the ones obtained with the susceptible Kisumu females. Cinnamaldehyde significantly repelled KdrKis and Acerkis females at both concentrations and was significantly more repellent at C2 against KdrKis females, with 74.2% to 90% escaped females, than on AcerKis and Kisumu females , with only 32.1% to 65.3% escaped females ($P<0.0001$). Cuminaldehyde significantly repelled both resistant females with 41% to 64.9% escaped KdrKis females and 35.2 % to 58.8% escaped AcerKis females at C2 ($P<0.0001$). Cuminaldehyde was significantly more repellent on KdrKis than Kisumu females (about 25% escaped females) ($P= 0.02$). Carvacrol and geraniol were significantly repellent on females KdrKis only, with 30.1% to 53.8% escaped females ($P<0.0001$). This was not significantly different from results obtained on Kisumu females (25% to 48% escaped females). These two compounds were not efficient against AcerKis females (no more than 20% escaped females).

Irritancy assay:

Permehrin did not induce behavioural responses of KdrKis and AcerKis females when tested at C1 and was then significantly more irritant against Kisumu females (31.1% to 55% escaped females). On the contrary, permethrin significantly irritated females KdrKis and AcerKis when tested at C2, with 16.7% to 57.4% escaped females. This was not different from the escaped percentages obtained with Kisumu females (35.2% to 58.8%). DEET and the four compounds induced a significant behavioural response of KdrKis and AcerKis females when tested at C1 ($P<0.0001$). Carvacrol was significantly less irritant against resistant females $P<0.001$), with only about 45.3% escaped KdrKis females and about 49.3% escaped AcerKis females, compared to Kisumu females (84.3%). Geraniol was on the contrary more irritant on KdrKis females, with about 73.4% escaped females compared to Kisumu (45.9%) ($P=0.003$).

Toxicity assay:

Permethrin was significantly less toxic to KdrKis females than Kisumu females when tested at both concentrations: at C1, about 2.7% killed KdrKis females while 93.8% Kisumu females were killed ($P=0.01$); and at C2, about 63.2% KdrKis females were killed while about 96.8% Kisumu females were killed. Permethrin also significantly killed less AcerKis females (66.1%) compared to Kisumu females at C1 ($P<0.01$), but AcerKis females were as susceptible to permethrin as Kisumu females at C2. DEET significantly killed less KdrKis females (about 20.9%) than Kisumu an AcerKis females (about 98%) at C2 ($P<0.0001$). Only cinnamaldehyde was significantly toxic on KdrKis and AcerKis females, and was toxic only when tested at C2 $P<0.0001$). With about 93.9% killed KdrKis females and about 89.9% killed AcerKis females, the resistant strains were significantly more susceptible to cinnamaldehyde than Kisumu females, with a mortality rate of about 45.9% ($P<0.0001$).

Experiment 3:

The last part of the study consisted in testing whether the promising compounds, carvacrol, geraniol, cuminaldehyde and cinnamaldehyde were effective against Kisumu females on a bed net when a bait was present, in order to simulate field conditions.

Residual efficacy on the nets:

See Figure 4, supplementary material

To determine if the four product carvacrol, geraniol, cuminaldehyde and cinnamaldehyde would be effective on netting during the 8 hours of duration of the tunnel experiment, we tested their residual effect using irritancy test. Geraniol and cinnamaldehyde remained significantly irritant 9 hours after impregnation ($P<0.05$), which percentages of escaped females ranging from 30% up to 60%. However, the percentages of escaped females decreased significantly, from 50% between 0 and 9 hours for cuminaldehyde, going from 60% escaped females down to 20%. The percentages of irritated females by carvacrol were significant 3 and 6 hours after impregnation (about 40% escaped females) but were not when tested just after impregnation (0 hours) nor 9 hours after. Carvacrol and cinnamaldehyde were still significantly toxic up to 9 hours after impregnation, with percentages of killed females of at least 40% for carvacrol and 60% for cinnamaldehyde ($P<0.01$). These two compounds were also significantly producing more knock down effects than controls with percentages from 100% (0 hours after impregnation) to 70% (9 hours after impregnation) knock down females. Cuminaldehyde lost toxicity and knock down effects after 3 hours and geraniol was not toxic neither responsible for knock down effects.

Tunnel experiment:

See Table 4, supplementary material

Permethrin, significantly inhibited crossing of the net (prevented 26.3% to 36.3% females from crossing the net), blood feeding (78.1% to 84.65% blood meal inhibition) and killed about 62% more females compared to control ($P<0.0001$). All but geraniol significantly inhibited blood meal uptake and significantly killed females Kisumu ($P<0.01$). Cuminaldehyde was the most efficient of the four compounds tested, as it inhibited up to 43.6% crossing, inhibited up to 69.1% blood meal uptakes and killed about 22.8% of the females tested ($P<0.0001$). Carvacrol and cinnamaldehyde did not inhibited crossing but significantly inhibited about 22% blood feeding and killed between 12.8% and 29.8% of the females tested.

Discussion

Cuminaldehyde, carvacrol and cinnamaldehyde are potential alternatives to pyrethroids for impregnating bet nets.

We identified 4 compounds which best characterized the effects of the four EO, citronella, cumin, thyme and cinnamon. These four compounds were geraniol, cuminaldehyde, carvacrol and cinnamaldehyde. These compounds could be the one responsible for the repellency or toxic effects found for the EO when these we tested. The compounds were also efficient when tested against resistant strains KdrKis (resistant to pyrethroids) and AcerKis (resistant to OP and CX) of *An. gambiae*. However, the three effects: repellency, irritancy and toxicity of the products were different depending on which population they were tested on. All but geraniol showed very good efficiency to inhibit blood meal uptake and to kill females from the susceptible reference strain Kisumu, when impregnated on a net, in simulated field conditions. However, these products were not tested at the same dosage (their ratio in their respective EO was not the same) so we could not directly compare their relative efficacy. But the concentrations tested (between 0.023 and 0.079 mg/cm^2) were about ten times smaller than the concentration tested for permethrin ($0.1mg/cm^2$). Therefore we can envisage testing higher concentrations of these products using tunnel tests. Geraniol on the contrary seemed to have attracted females instead of repelling them, and did not kill them. Therefore, this compound will not be further tested and only carvacrol, cuminaldehyde and cinnamaldehyde are good candidates to be tested in parallel with permethrin on the field.

Cuminaldehyde was the most efficient of the products tested to inhibit blood meal uptakes by females *An. gambiae* in field simulated conditions, and was the most toxic after permethrin (tested at $0.1mg/cm^2$). Curiously, cuminaldehyde had lost toxicity 3 hours after impregnation on a net, when we tested its persistence on a net. Differences in toxicity between experiments might here be explained by a higher concentration of product on a net than on a paper, because of the smaller surface of contact. The gain of toxicity was visible when cuminaldehyde was tested on nets in residual tests and tunnels. Cuminaldehyde was also found toxic for larvae of *An. simplex* [62].

The results we obtained for carvacrol are in accordance with previous researches, when carvacrol was found repellent to the agricultural pest *Spodoptera litura* [63], toxic to the fungus *Botrytis cinerea* [64] and toxic to the larvae of the nematod *Anisakis simplex* [62]. Also, the toxicity of carvacrol, found only when tested on a net, indicates that a higher concentration of carvacrol than used in our tests ($0.0024mg/cm^2$) could be toxic. Per square centimetre of net, the surface treated is smaller compared to paper because of holes. It is possible that the quantity of compound is higher in these smaller surfaces so the females were in contact with higher doses of carvacrol, and were then intoxicated.

Contrary to carvacrol and cuminaldehyde, the toxicity of cinnamaldehyde decreased on the net compared to paper, indicating that higher doses of cinnamaldehyde might not increase the toxicity, or that a direct contact does not induce toxicity in adult *An. gambiae*. Cinnamaldehyde was already found highly larvicidal on *Ae. Aegypti*, when tested at very low doses (LD90 at 48ppm) [65]. This product also showed fumigant and antifeedant effects on the grain storage pest *T. castaneum* [66] and was found toxic against houseflies, but after topic application on the thorax of the adult fly (LD50:1.04µg/insect) [67].

Unlike our results on *An. gambiae*, geraniol was found to be toxic for the housefly *Musca domestica*. However, these results were obtained after topic application of geraniol on the thorax of adult flies (LD50: 1.93 µg/insect) [67]. and for larvae of *An. simplex* [68] and *Aedes albopictus* [69]. This also indicates that efficiency of plant EO compounds could depend on the insect species and on the insect development stage and especially on the quantity the insect is exposed to.

The mode of action of the compounds tested here should be further studied. First, to determine how they penetrate within the insect body: ingestion, inhalation or cuticle absorption. For instance, toxicity can be caused by toxic vapors or by contact with products [70]. This last possibility could be applied to carvacrol. Indeed, carvacrol was significantly toxic to the females *An. gambiae* when applied on the bed net in simulated field conditions while it was not in the toxicity assays. If a higher concentration in direct contact with the females, caused toxicity, maybe carvacrol is toxic by contact.
Second, and with regards for human health, it would be interesting to look at target sites of the compounds we identified. Many studies looked at the toxicity of EO and their compounds, and pointed out an obvious neurotoxic

mode of action in insects [46, 71]. For instance, a neurotoxic mode of action which characterizes some EO compounds, (ie eugenol and cinnamic alcohol) is the binding of H-octopamin in the Octopaminergic nervous system of the fruit fly and the American cockroach [72, 73]. Other compounds, Thymol for instance, binds to GABA receptors associated with chloride channels located on the membrane of postsynaptic neurons and disrupts the functioning of GABA synapses in *D. melanogaster* and in humans[47]. Also, certain EO monoterpenes are inhibitors of AchE in vitro [74] and linalool, a thyme compound without effects in our study, was found also to be an AchE inhibitor [75]. One of the products identified here could have neurotoxic mode of actions: Carvacrol, found to be less repellent and less irritant against the resistant AcerKis females, could act as AchE-1 inhibitor or similar activity, which a mutation of Ache1 would have protected the mosquitoes from. The measure of AchE1 activities in presence of carvacrol on the three strains would allow us to investigate if Ache1 from AcerKis is less inhibited by these molecules. On the contrary, cuminaldehyde and cinnamaldehyde showed more repellency and toxicity efficiency on both resistant strains than Kisumu, indicating that apparently these compounds do not affect the VdpNaC or the AchE activity but they might affect mechanisms which are more susceptible in resistant strains than Kisumu strain. Also, we could not identify here if the compounds would act on the octopaminergic system. It would be interesting to test cinnamaldehyde, as it is the case of its related compound, cinnamic alcohol. These products, if neurotoxic for insects could also be for humans therefore their toxicity to humans should be assessed before to be used for public health.

In order to reduce the risks of emergence of populations of *An. gambiae* with resistance or multiple resistances to the future alternative to pyrethroids, it would be interesting to look at the effects of mixtures of 2 compounds. With different modes of actions (ie repellency and irritancy) and different target sites (ie Ache1 and VdpNaC), such mixture could permit to limit the risks of resistance by mutation or insensitivity to one particular product, which acts on a specific target, as the idea was already suggested concerning pyrethroids and other insecticides [36].

To complete the study and find potential alternatives to currently used pyrethroids, it remains to assess the efficacy of our selected compounds in the field. Notably, the laboratory tunnel experiment could be useful to test higher concentration of the three selected products, against Kisumu females and resistant strains from the two existing molecular forms (in this study the behaviour of Savanna *An. gambiae* only was studied) and other mosquitoe species before field experiments. Also, we found that these compounds could be fixed on a net but remain to be formulated in order to reduce their volatility and increase their residual efficacy. The stability of these molecules should also be studied and modified if necessary: For instance pyrethrum, from which originate pyrethrin, was labile because of sunlight. They were synthetically modified to be more stable at sunlight, giving birth to the pyrethroids family currently used in agriculture and public health [17].

We cannot generalize the characterization of plant EO and their effects on mosquitoes.

These results confirmed the efficient repellency, irritancy and toxic effects of the citronella, cumin, thyme and cinnamon EO previously found (deletre et al submitted) on mosquitoes. In most cases, in literature, only the toxicity of the complete EO, or the major compounds was studied [63, 70, 76]. Generally major compounds were found to poorly the biological properties found for the EO [76], thus the question of a synergistic action between compound is usually arisen. The results showed here demonstrated that indeed the major compounds alone generally have similar effect than the EO; but depending on how the compounds are mixed, the effects obtained were different. We found that all compounds, including the minor ones, modulated the effects of the most active one. For instance the mixture of all compounds increased the effects of the cuminaldehyde in the cumin mixture and similar events occurred with the cinnamon mixture. The modulation of the major compounds effects by minor ones was already suggested a few years ago [77].

In our study, repellent and irritant actions of an EO were the property of one (cuminaldehyde from cumin EO) to three (citronellal, geraniol and citronellol from citronella EO) main compounds and toxicity resulted more from the mixture between compounds. The compounds were not necessarily toxic themselves (thyme EO) and minor compounds seemed to be as important as main compounds in the effectiveness of the EO (citronella EO). However, some molecules possessed the three effects and reflected alone the effects of the EO (cinnamon EO). Therefore, we cannot generalize the characterization of plant EO and their effects on mosquitoes: it depends on the composition of the EO. Also it was determined that a compound has not the same efficiency on different insect species [63, 70] and we could observe these differences when we tested three populations of *An. gambiae*: we observed different efficacy while the only difference between these strains (from the same species and from the same molecular form S) is one mutation on one gene (refer to introduction). The investigations of toxicity effect of EO and their main compounds against stored

product pests the red flour beetle *Tribolium castaneum* and the maize weevil beetle *Sitophilus zeamais* [66] for instance, have led to the same conclusions.

Citronella and thyme compounds are potential alternative repellents for personal protection against mosquitoes.

The low mammalian toxicity of EO has already been assessed [63, 78] but some EO contain photoactive molecules like furocoumarins, and these EO are cytotoxic and mutagenic under light exposure. However, they are not toxic or mutagenic by themselves in the dark [63]. Citronella is a genus of *Cymbopogons* family. The phototoxicity of its sister species *C. citratus* was proven [67] but there are no evidences that citronella EO we tested here (*C. winterianus*) show the same properties. In this study, we could identify which compounds were not responsible for the toxicity of citronella EO, but we could not determine what caused this toxicity. It would be interesting to identify where the phototoxicity in this family comes from because there are no evidence that such mutagenic properties would not affect humans. Also, we tested here only major compounds separately and the mixture of these. A minor compound, which ratio is lower than 3%, could be responsible for EO toxicity, alone or mixed.

Compounds from citronella can be potential alternatives to repellents, especially since they showed no toxicity on mosquitoes when tested alone and the efficacy of their mixture equals the one of the EO, without toxicity. Thyme compounds were also non-toxic when not mixed. Therefore all these compounds are potential alternatives to repellents to be used for personal protection, as the toxicity can be removed; at least against mosquitoes. The separation of the three effects of EO, repellency, irritancy and toxicity could be also interesting to be used against pests in agriculture. Other studies have showed the efficacy of plant EO against crop pests such as cumin EO against the cotton aphid *Aphis gossypii* and the spider mite *Tetranychus cinnabarinus* [79]. Also linalool and cinnamaldehyde were revealed to be particularly repellent against the white fly *Bemisia tabacci* (Deletre et al. unpubl.). This agricultural pest and disease vector for tomatoes, which is also resistant to insecticides such as pyrethroids and organophosphates [80].

DEET is less toxic to KdrKis resistant *An. gambiae* than AcerKis resistant and susceptible Kisumu.

Results on KdrKis (resistant to pyrethroids) were validated as the effect of permethrin was effectively reduced compared to Kisumu. However our results were unexpected concerning the irritant effect of permethrin. Because of its high toxicity, Kisumu females could not escaped the cylinders in the irritancy tests because they were knock down 1 min after exposure, while KdrKis females were not. Therefore we could not observe its higher irritancy on susceptible females compared to KdrKis females. The 100% toxicity observed when permethrin and DEET were tested on the susceptible Kisumu females are in accordance with previous results observed [81].

Interestingly and surprisingly, we obtained a significant reduction of toxicity by DEET against Kdr-Kis. This study is the first in which DEET was tested on the resistant Kdr *An. gambiae*. DEET was identified as AchE inhibitor when tested on in vitro preparations [24]. We would have expected DEET to be less toxic for the resistant females AcerKis, resistant to insecticides which target is AchE-1, and we would have expected no differences in sensitivity between Kisumu and KdrKis as these latest were not known to resist AchE inhibitors.These unexpected results deserve further attention to determine how the repellent DEET works exactly on Kdr-Kis females, AcerKis females and other resistant types. At last it can be notified that populations used were not conditioned to any odors or insecticides therefore this decrease cannot be explained by learning, as it was observed in recent studies when DEET was tested on *Ae. aegypti* [41]. Also, if further results reveal that KdrKis resistant were also resistant to DEET, this study have provided interesting potential alternatives to repellents, with non-toxic but repellent and irritant compounds from citronella, thyme and cumin EO (see above).

Conclusion

The results of this study are particularly innovating. Spatial repellency, contact irritancy and toxicity of EO have been separated and characterized as properties from some of the major EO compounds. We reached our goals with the identification of three potential alternatives to pyrethroids: carvacrol, cuminaldehyde cinnamaldehyde. These three compounds showed efficient repellency, irritancy and toxicity effects against susceptible and resistant females *An. gambiae*, a major malaria vector. However, these products remain to be further tested, particularly effects on human health, to be able to replace pyrethroids. These alternatives are promising issues for public health, with long lasting bed nets, personal protection, but may also be interesting for agriculture protection with crop nets. Last but not least, we identified for the first time the loss of toxic effect of DEET on resistant KdrKis females *An. gambiae*, which is very intriguing. The efficacy and use of the most efficient repellent used nowadays (DEET) is questioned and have to be further studied.

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Supplementary material

Table 2: Ratios and quantities of the compounds of citronella, cumin, thyme and cinnamon EO used in this study.

EO	Composition (%)	Quantity tested (mg/cm^2) C1	C2
Citronella *Cymbopogon winterianus*	35 % citronellal	0.0035	0.0347
	23 % geraniol	0.0023	0.0225
	12 % citronellol	0.0012	0.0120
	3,3 % limonene	0.0003	0.0033
	3,5 % geranyl-acetate	0.0004	0.0035
	76 % Total (mix)	**0.0100**	**0.1000**
Cumin *Cuminum cyminum*	30 % cuminaldehyde	0.0030	0.0301
	12 % β-pinene	0.0012	0.0122
	12 % γ-terpinene	0.0012	0.0116
	9,7 % *p*-cymene	0.0010	0.0097
	64 % Total (mix)	**0.0100**	**0.1000**
Thyme *Thymus vulgaris*	31 % thymol	0.0031	0.0305
	24 % carvacrol	0.0024	0.0237
	8,4 % α-terpinene	0.0008	0.0084
	4 % Linalool	0.0004	0.0040
	3,5 % β-caryophyllene	0.0004	0.0035
	70 % Total (mix)	**0.0100**	**0.1000**
Cinnamon *Cinnamomum zeylanicum*	79 % cinnamaldehyde	0.0079	0.0785
	9,6 % 2-metoxy-cinnamaldehyde	0.0010	0.0096
	3,1 % cinnamyl-acetate	0.0003	0.0031
	91 % Total (mix)	**0.0100**	**0.1000**

The used quantities are expressed in mg/cm^2 of chromatograph paper (first and second experiment) or net (third experiment). The composition of the four EO was identified by Gas Chromatography and Mass Spectrometry.

Fig. b

Fig. a

Fig. c

Fig. e

Fig. d

Fig. f

Figure 1 : a. paper impregnation; b. net impregnation c. High throughput screening system (HiTSS) used for spatial repellency tests; d. WHO test kit used for contact irritancy tests; e. WHO test kit used for toxicity tests; f. Tunnel apparatus used to simulate field conditions (courtesy of V. Corbell, IRD, France).

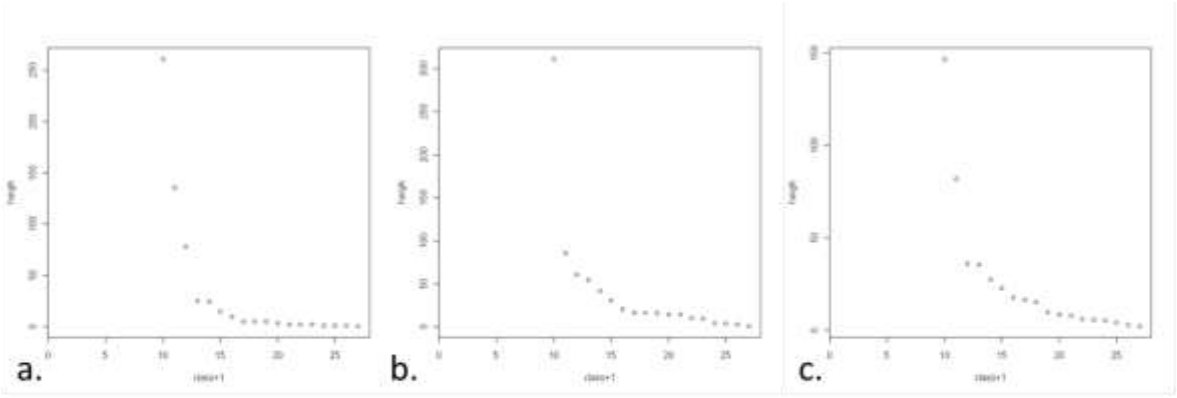

Figure 2 **Interclass variance with Ward's method represented by the height branch between two nodes of the dendrogramm (a : spatial repellent, b. Contact irritancy, c. Toxicity).**

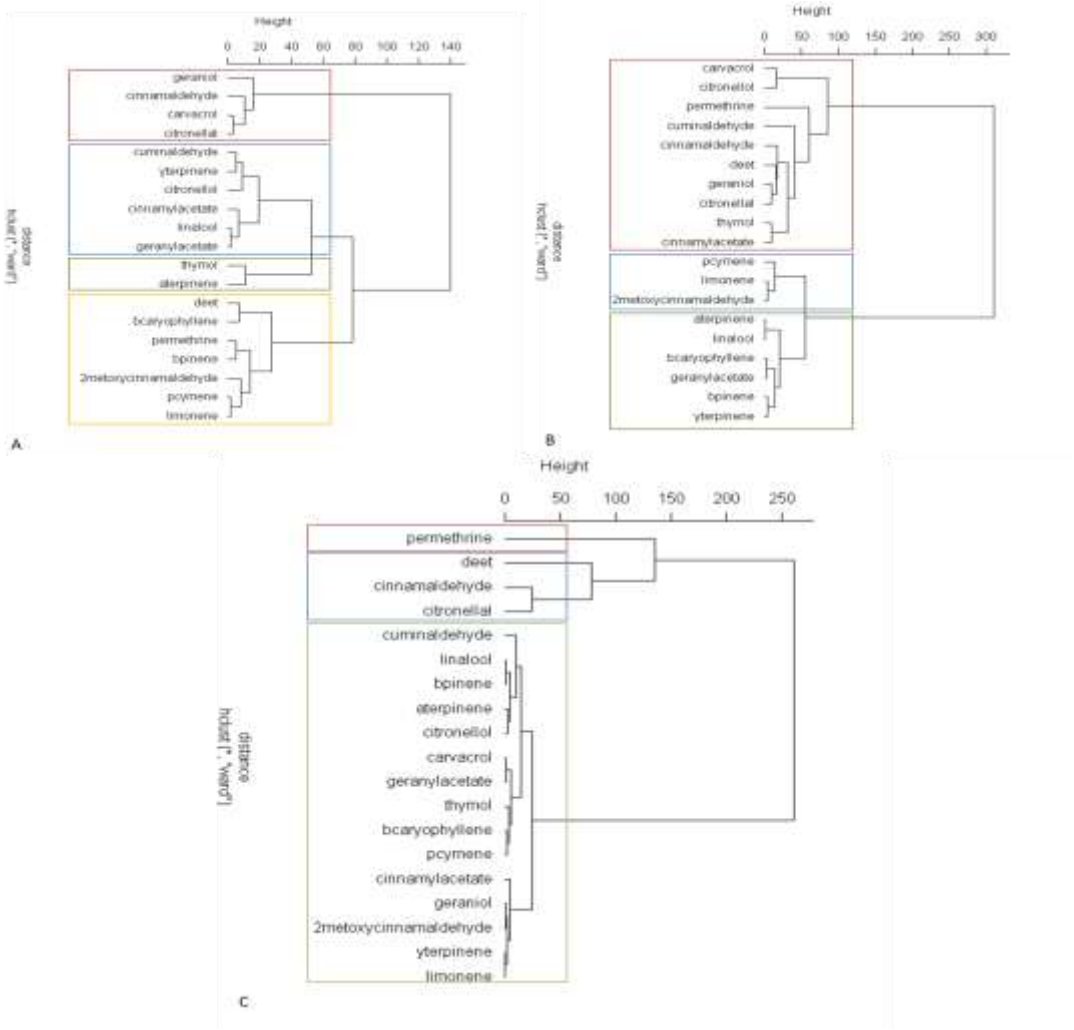

Figure 3 : Hierarchical ascendant classification (HAC) based on Ward's algorithm. a. Repellency, b. irritancy and c. toxicity effects of the EO compounds, DEET and permethrin on non-blood fed 2-5 days old females *Anopheles gambiae* Kisumu. The number of classes was based on the interclass variance (refer to Figure 2).

Table 2: Repellency, irritancy and toxicity effects of DEET, permethrin and selected plent EO compoundcompounds on non-blood fed 2-5days old females *Anopheles gambiae ss* from the sensitive reference strain Kisumu.

PRODUCT	REPELLENCY %				IRRITANCY %				MORTALITY %			
	C1		C2		C1		C2		C1		C2	
	N	%(confidence interval)	N	%(confidence interval)	N	%(confidence interval)	N	%(confidence interval)	N	%(confidence interval)	N	%(confidence interval)
Permethrin	65	-0,2 (-5.3 - 4.9)	63	6,3 (-1.5 - 14)	67	**43,0 (31.1 - 55)**	64	17,1 (7 - 27.2)	67	**93,8 (89.7 - 97.8)**	55	**96,8 (96.8 - 96.8)**
DEET	58	-3,3 (-8 - 1.4)	69	15,0 (5.2 - 24.7)	66	7,6 (-0.2 - 15.5)	70	**54,1 (42.6 - 65.7)**	45	2,6 (-3.4 - 8.6)	44	**98,2 (98.2 - 98.2)**
Thym-oil	72	-1,3 (-5.1 - 2.5)	65	**51,4 (39.3 - 63.4)**	76	20,1 (9.5 - 30.6)	78	**59,0 (49 - 69)**	66	5,3 (-2.5 - 13.2)	58	**79,5 (70.6 - 88.4)**
Thym-mix	63	8,6 (-0.1 - 17.2)	62	**83,0 (75.2 - 90.9)**	67	**35,6 (23.8 - 4** *	62	**38,7 (26.6 - 50.8)**	54	7,5 (-0.2 - 15.2)	61	**83,5 (74.6 - 92.4)**
thymol	58	16,6 (6.2 - 27)	58	-2,4 (-5.7 - 1 *	59	**25,4 (13 - 37.7)**	58	**34,7 (21,8 - 47,5)**	60	1,2 (-5.1 - 7.5)	45	-1,0 (-7 - 5) *
carvacrol	72	9,4 (1.7 - 17)	68	**36,6 (25 - 48.2)**	68	**50,0 (38.2 - 6** *	71	**84,3 (77,4 - 91,2)**	70	4,3 (-0.5 - 9)	56	0,0 (0 - 0) *
a-terpinene	63	**25,7 (14.4 - 3** *	68	4,3 (-2.4 - 11 *	69	1,6 (-2.4 - 5.6)	75	10,7 (3,3 - 18, *	71	1,4 (-1.3 - 4.1)	66	4,5 (-0.5 - 9. *
linalool	63	12,7 (4.5 - 20.9)	68	17,6 (8.6 - 26 *	71	1,6 (-4.3 - 7. *	58	10,1 (0,8 - 19, *	64	3,1 (-1.1 - 7.4)	63	3,2 (-1.2 - 7. *
b-caryophyllene	59	-8,7 (-14.3 - -3.1)	62	10,4 (-0.2 - 21 *	63	4,3 (-4.4 - 12.9)	65	20,8 (9,6 - 32) *	59	0,1 (-3.2 - 3.4)	53	2,2 (-2.9 - 7. *
Citronella-oil	56	-1,3 (-8.1 - 5.4)	69	**54,3 (42,7 - 65,8)**	68	33,0 (21.1 - 44.8)	66	**60,3 (50.5 - 70.2)**	69	5,6 (-1 - 12.3)	55	**75,1 (64.2 - 86)**
Citronella-mix	65	**32,9 (21 - 44.** *	56	**76,2 (67.3 - 85.2)**	71	**34,2 (23 - 45.4)**	78	**82,2 (74.2 - 90.2)**	68	-3,6 (-6.5 - -0.8)	68	-0,2 (-5.6 - 5. *
citronellal	70	8,9 (0.3 - 17.4)	66	**40,1 (28.1 - 52.2)**	67	24,4 (12.5 - 36.4)	73	**52,2 (42,1 - 62,3)**	54	-3,2 (-3.2 - -3.2)	61	21,4 (10.6 - 3 *
geraniol	62	5,1 (-2.7 - 13)	60	**50,5 (38 - 63.1)**	66	16,5 (5.2 - 27.7)	67	**45,9 (34,2 - 57,5)**	59	-1,0 (-5.6 - 3.6)	60	-1,1 (-5.6 - 3. *
citronellol	51	10,6 (1.1 - 20)	60	25,2 (13.8 - 36.6)	70	**46,4 (35.3 - 57.5)**	66	**68,6 (60,7 - 76,5)**	55	0,1 (-4.9 - 5)	61	6,3 (-1.2 - 13 *
limonene	63	-0,1 (-4.4 - 4.2)	61	-3,3 (-3.3 - -3 *	70	-3,1 (-11.3 - 5.1)	71	-3,3 (-11,3 - 4 *	66	-1,8 (-4.7 - 1.2)	64	-1,7 (-4.8 - 1. *
geranyl-acetate	61	13,1 (4.6 - 21.6)	71	15,5 (7.1 - 23 *	67	6,8 (-1.7 - 15.4)	57	21,7 (9,9 - 33, *	58	3,4 (-1.2 - 8.1)	58	0,0 (0 - 0) *
Cumin-oil	64	4,7 (-0.5 - 9.9)	67	**37,3 (25.7 - 48.9)**	70	7,9 (-1.2 - 17)	80	**79,4 (72.8 - 86)**	63	-2,9 (-2.9 - -2.9)	59	**34,4 (22.1 - 46.8)**
Cumin-mix	62	0,0 (0 - 0)	62	**30,6 (19.2 - 42.1)**	76	4,8 (-2.1 - 11.7)	79	**41,1 (30.1 - 5** *	62	-0,2 (-3.4 - 2.9)	62	9,4 (1.6 - 17 *
cuminaldehyde	62	1,6 (-1.5 - 4.7)	59	**25,4 (14.3 - 36.5)**	71	10,4 (1.1 - 19.6)	68	**77,5 (69,4 - 85,6)**	65	0,0 (0 - 0)	63	11,1 (3.4 - 18 *
b-pinene	75	3,9 (-1.2 - 9)	71	2,8 (-1.9 - 7. *	66	11,2 (1.1 - 21.3)	81	15,6 (6 - 25,3) *	59	3,4 (-1.2 - 8)	51	3,9 (-1.4 - 9. *
g-terpinene	68	4,5 (-1.1 - 10.1)	67	21,0 (11 - 31)	73	8,9 (-0.4 - 18.1)	78	12,7 (3,2 - 22, *	72	-1,6 (-1.6 - -1.6)	69	-1,6 (-1.6 - -1 *
p- cymene	71	-0,1 (-4 - 3.7)	68	-1,5 (-4.3 - 1. *	73	9,4 (0.2 - 18.7)	65	-6,5 (-11,6 - - *	56	1,8 (-1.7 - 5.3)	70	1,4 (-1.4 - 4. *
Cinnamon-oil	60	8,5 (0.9 - 16.1)	69	**84,0 (75,7 - 92,3)**	74	25,8 (15.2 - 36.3)	69	**90,4 (85.6 - 95.2)**	60	3,3 (-1.2 - 7.9)	66	**43,9 (32 - 55.9)**
cinnamon-mix	68	**12,4 (3.3 - 21.5)**	56	**66,2 (54,4 - 78)**	71	**56,7 (46.4 - 67)**	69	**60,2 (50.3 - 70.2)**	63	3,1 (-2.9 - 9.1)	55	**76,7 (66.2 - 8** *
cinnamaldehyde	73	16,5 (7.8 - 25.3)	79	**43,0 (32.1 - 5** *	81	22,0 (12.3 - 31.7)	79	**63,2 (52.9 - 73.4)**	57	-1,5 (-1.5 - -1.5)	57	**45,9 (32.9 - 58.8)**
2-metoxy-cinnamaldehyde	62	-6,8 (-12.2 - -1.5)	58	-3,0 (-10.3 - 4 *	59	0,3 (-9.9 - 10.6)	58	-1,0 (-11.1 - 9 *	57	-1,1 (-6.9 - 4.7)	47	0,0 (-7 - 7) *
cinnamyl-acetate	65	7,6 (-0.8 - 16)	68	15,8 (6 - 25.7 *	66	29,2 (17.2 - 41.2)	79	**43,5 (32.6 - 5** *	57	-3,6 (-3.6 - -3.6)	54	-3,6 (-3.6 - -3 *

Results are displayed for the concentrations C1 and C2 (refer to Table1). This table display Abbott's corrected percentages with confidence intervals (central limit theorem) between quotes.

Numbers in bold characters are significantly different from their respective controls (P<0,05). * Percentages are significantly different from the percentage found with the EO from which the product originated (P<0,05).

Table 3: Repellency, irritancy and toxicity effects of DEET, permethrin and selected EO compoundcompounds on females *Anopheles gambiae ss* from the sensitive reference strain Kisumu and from the resistant strains KdrKis and AcerKis.

REPELLENCY												
	C1						C2					
	Kisumu		KdrKis		AcerKis		Kisumu		KdrKis		AcerKis	
PRODUCT	N	%(confidence interval)	N	%(confidence interval)	N	%(confidence interval)	N	%(confidence interval)	N	%(confidence interval)	N	%(confidence interval)
Permethrin	65	-0,2 (-5.3 - 4.9)	67	4,3 (-2 - 10.6)	48	2,7 (-4.1 - 9.6)	63	6,3 (-1.5 - 14)	71	11,0 (2.9 - 19.1)	52	8,0 (-0.7 - 16.7)
DEET	58	-3,3 (-8 - 1.4)	59	20,1 (9.2 - 31)	58	11,9 (2.6 - 21.2)	69	15,0 (5.2 - 24.7)	61	**29,2 (17.4 - 40.9)**	56	**85,7 (77.6 - 93.8)** *
carvacrol	72	9,4 (1.7 - 17)	64	11,2 (3.1 - 19.3)	58	5,7 (-2.2 - 13.5)	68	**36,6 (25 - 48.2)**	67	**42,0 (30.1 - 53.8)**	57	-1,2 (-6 - 3.6) *
geraniol	62	5,1 (-2.7 - 13)	72	3,1 (-3.2 - 9.5)	50	**22,1 (9.4 - 34.8)**	60	**50,5 (38 - 63.1)**	65	**41,0 (28.9 - 53.1)**	51	9,7 (-0.7 - 20.2) *
cuminaldehyde	62	1,6 (-1.5 - 4.7)	58	24,7 (12.6 - 36.8) *	65	8,5 (0.6 - 16.5)	59	**25,4 (14.3 - 36.5)**	64	**52,9 (41 - 64.9)** *	69	**47,0 (35.2 - 58.8)**
cinnamaldehyde	73	16,5 (7.8 - 25.3)	63	**36,2 (24 - 48.4)**	56	**37,7 (24.9 - 50.5)**	79	**43,0 (32.1 - 54)**	62	**82,1 (74.2 - 90)** *	64	**53,1 (40.9 - 65.3)**

IRRITANCY												
	C1						C2					
	Kisumu		KdrKis		AcerKis		Kisumu		KdrKis		AcerKis	
PRODUCT	N	%(confidence interval)	N	%(confidence interval)	N	%(confidence interval)	N	%(confidence interval)	N	%(confidence interval)	N	%(confidence interval)
Permethrin	67	**43,0 (31.1 - 55)**	67	3,0 (-2 - 7.9) *	57	10,1 (-0.8 - 21)	64	17,1 (7 - 27.2)	68	**45,6 (33.7 - 57.4)**	74	**27,9 (16.7 - 39.1)**
DEET	66	7,6 (-0.2 - 15.5)	67	22,6 (11.3 - 33.8)	67	16,5 (7.3 - 25.7)	70	**54,1 (42.6 - 65.7)**	70	**71,2 (62.1 - 80.3)**	71	**43,6 (32.1 - 55.2)**
carvacrol	68	**50,0 (38.2 - 61.8)**	69	0,1 (-7.4 - 7.7) *	65	13,9 (5.2 - 22.7) *	71	**84,3 (77.4 - 91.2)**	67	**45,3 (33.4 - 57.2)** *	65	**49,3 (37.2 - 61.5)** *
geraniol	66	16,5 (5.2 - 27.7)	81	24,8 (14.5 - 35.2)	66	19,6 (9.1 - 30.2)	67	**45,9 (41.4 - 50.4)**	79	**73,9 (65.7 - 82)** *	73	**41,8 (30.4 - 53.3)**
cuminaldehyde	71	10,4 (1.1 - 19.6)	66	20,2 (9.5 - 31)	79	9,6 (1.9 - 17.2)	68	**77,5 (69.4 - 85.6)**	80	**63,0 (53 - 73)**	77	**46,3 (35.2 - 57.5)**
cinnamaldehyde	81	22,0 (12.3 - 31.7)	67	23,8 (12.7 - 34.9)	65	11,7 (1.4 - 21.9)	79	**63,2 (52.9 - 73.4)**	68	**70,4 (60.6 - 80.3)**	56	**51,1 (38.4 - 63.8)**

TOXICITY												
	C1						C2					
	Kisumu		KdrKis		AcerKis		Kisumu		KdrKis		AcerKis	
PRODUCT	N	%(confidence interval)	N	%(confidence interval)	N	%(confidence interval)	N	%(confidence interval)	N	%(confidence interval)	N	%(confidence interval)
Permethrin	67	**93,8 (89.7 - 97.8)**	48	2,7 (-4.1 - 9.6) *	56	**66,1 (53.7 - 78.5)** *	55	**96,8 (96.8 - 96.8)**	48	**63,2 (49.8 - 76.5)** *	57	**100,0 (100 - 100)**
DEET	45	2,6 (-3.4 - 8.6)	60	-2,0 (-5.3 - 1.2)	53	-3,4 (-3.4 - -3.4)	44	**98,2 (98.2 - 98.2)**	57	20,9 (9.7 - 32) *	54	**96,6 (96.6 - 96.6)**
carvacrol	70	4,3 (-0.5 - 9)	50	3,3 (-3.3 - 9.8)	56	-6,9 (-12.8 - -1)	58	0,0 (0 - 0)	70	-2,7 (-2.7 - -2.7)	52	-10,3 (-14.1 - -6.6)
geraniol	59	-1,0 (-5.6 - 3.6)	60	1,7 (-1.6 - 4.9)	50	-2,6 (-9.2 - 4)	60	-1,1 (-5.6 - 3.5)	65	3,1 (-1.1 - 7.3)	51	1,2 (-7 - 9.3)
cuminaldehyde	65	0,0 (0 - 0)	65	1,3 (-2.9 - 5.5)	59	-7,4 (-10.7 - -4.1)	63	11,1 (3.4 - 18.9)	54	3,8 (-2.3 - 9.9)	50	30,9 (17.4 - 44.5) *
cinnamaldehyde	57	-1,5 (-1.5 - -1.5)	54	0,0 (0 - 0)	54	0,2 (-3.4 - 3.8)	57	**45,9 (32.9 - 58.8)**	49	**93,9 (87.2 - 100.6)** *	47	**89,9 (81.9 - 97.9)** *

Results are displayed for the concentrations C1 and C2 (refer to table 1). This table display Abbott's corrected percentages with confidence intervals (central limit theorem) between quotes.

Numbers in bold characters are significantly different from their respective controls (P<0,05). * Percentages are significantly different from the percentage found with the Kisumu strain (P<0,05).

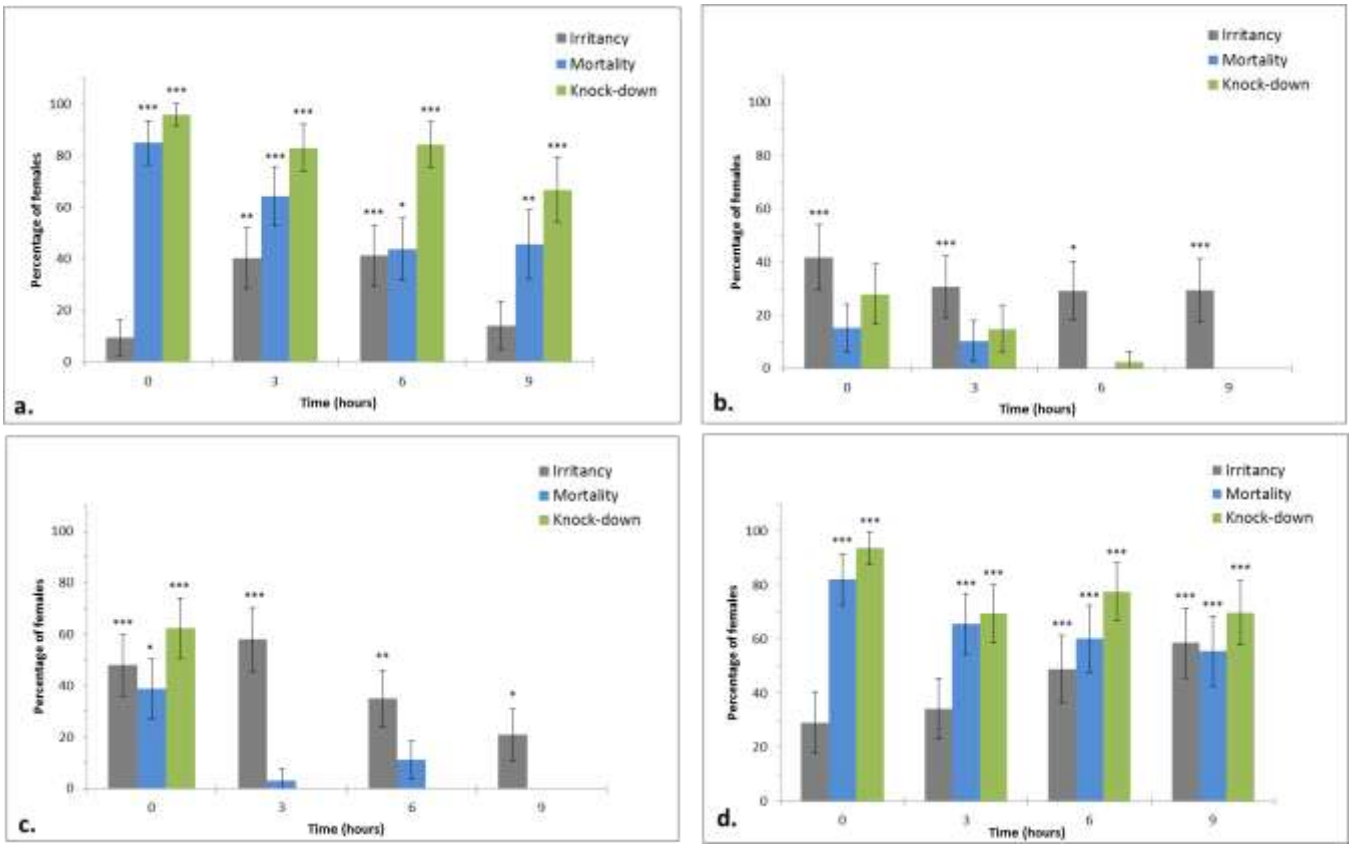

Figure 4: Percentages (corrected with Abbott's formula) +/- CI (central limit theorem) of non-blood fed females *An. gambiae* that are irritated (grey bars), killed (blue bars) and knocked down (green bars) by the four tested product 0, 3, 6 and 9 hours after the net was impregnated (a. Carvacrol, b. Geraniol, c. Cuminaldehyde and d. Cinnamaldehyde), *0,05<p<0,01; **0,001<P<0,01; ***P<0,001.

Table 4: Blood feeding inhibition, crossing inhibition and toxicity of permethrin, carvacrol, geraniol, cuminaldehyde and cinnamaldehyde on females Kisumu of *An. gambiae* when tested in field simulated conditions.

Compound	Quanity tested (µl/cm^2)	N	Crossing inhibition (%)	Blood feeding inhibition (%)	Toxicity (%)
permethrin	-0.1	362	31.2 (26.2 – 36.3)***	81.3 (78.1 – 84.6)***	62.5 (57.6 – 67.5)***
carvacrol	0.024	219	5.5 (0.5 – 10.6)***	23.3 (16.6 – 29.9)***	23.2 (17.1 – 29.4)***
geraniol	0.023	291	-8.3 (-11.2 - -5.4)**	-25.1 (-30 - -20.2)***	4.6 (1.2 – 8.1)
cuminaldehyde	0.03	277	37.8 (31.9 – 43.6)***	64.1 (59.1 – 69.2)***	22.8 (17.4 – 28.3)***
cinnamaldehyde	0.079	268	7.1 (2.3 – 11.9)***	22.5 (16.6 – 28.5)**	17.8 (12.8 – 22.8)***

***P<0.001, **0.001<P<0.01.